

Research Article

MICROBIOLOGICAL EVALUATION OF BOVINE FROZEN SEMEN SAMPLES IN WEST BENGAL, INDIA

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ABSTRACT: A total number of 860 French mini straws (0.25 ml) of frozen semen from 215 bulls from three different farms namely frozen semen bull station (FSBS), Harighata Farm (98), FSBS, Salboni (93) and Sperm Station, Beldanga (24) were evaluated for bacterial load by standard plate count (SPC) technique using soyabean casein digest agar and 1% plain agar media. Following incubation at 37°C for 72 hrs average colony forming unit (CFU) was estimated and bacteria were identified. Different micro-organisms identified in frozen semen samples were *Staphylococcus* spp., *Micrococcus* spp., *Escherichia coli*, *Pseudomonas* spp., *Corynebacterium* spp., *Proteus* spp., *Klebsiella* spp., *Bacillus* spp. other than *Bacillus anthracis* and *Streptococcus* spp. Several of these bacteria have been identified in association with breeding failure in cattle and warrants precautionary and preventive measures for successful breeding program.

Key words: Bull, Frozen Semen, Bacteria.

INTRODUCTION

Artificial insemination (AI) of cows and heifers with frozen semen is widely practiced in West Bengal. The hygiene status of semen is important for vitality of spermatozoa and for the fertility of inseminated cows. Presence of bacteria, fungi and viruses has been detected in semen samples that deteriorate semen quality, as well as, transmit the pathogen to next generation. The role of specific microbes in semen leading to reproductive disorder among dairy animals is well established. Despite

sanitary precautions, several ubiquitous and opportunistic microbes find their ways into semen during harvesting, processing and storage of semen (Sannat *et al.* 2015). A correlation was shown to exist between bacterial load and semen quality. Increase bacterial load can lead to deterioration sperm motility and viability (Shukla 2005). Addition of antibiotics to semen extenders prevents bacterial growth and may improve conception rates. The number of bacteria in semen can be decreased by keeping bulls clean and regular

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rinsing of the preputial cavity (Thibier and Guerin 2000). Hence, in the present investigation, the qualitative and quantitative estimation of bacteria was conducted in frozen semen samples of bovine and buffalo bulls.

MATERIALS AND METHODS

Source of samples

A total of 860 numbers of frozen semen straw (FSS) samples from 215 bulls were collected from three different frozen semen bull stations (FSBS) of West Bengal, namely, FSBS, Haringhata Farm (98 bulls), FSBS, Salboni (93 bulls) and Sperm Station, Beldanga (24 bulls). In this study, four straws were collected for each bull in four different quarters in 2014. The study was conducted as per OIE protocol at Infectious Abortion Scheme Laboratory (IAS lab) and Research Laboratory for Bacterial Diseases (RLBD), Institute of Animal Health & Veterinary Biologicals (R&T), Kolkata.

Estimation of Microbial load

Two media, namely, soyabean casein digest agar media (SCDA) (Hi-media) and 1% plain agar media (Hi-media) were used for bacterial enumeration in semen samples. Four numbers of frozen semen straws for each ejaculate were thawed and pooled to 1ml in sterile microcentrifuge tube and marked according to the identification number of the bull. Ten fold serial dilutions ($10^{-1}, 10^{-2}, 10^{-3}$) of the semen samples were prepared in dilution media containing 10g peptone, 5g NaCl, 9g Na_2HPO_4 , 1.5g KH_2PO_4 , 1000 ml distilled water. For each pooled and diluted semen three petri plates were inoculated with each diluted semen (10^{-3}); one plate remained as negative control in which only dilution media (1ml) was plated. Ten milliliter of SCDA containing 5% fetal bovine serum

(Hi-media) was plated and solidified for 1 hr. Then 5 ml of 1% plain agar media was poured into preformed SCDA plate and kept for 1 hr for drying. The inoculated plates were incubated at 37°C for 72 hrs when number of colonies in each plate was estimated, averaged for three plates for each and multiplied by dilution factor (10^3) to get colony forming unit (CFU)/ml of semen sample [$\text{CFU}/\text{ml} = \{(\text{P1} + \text{P2} + \text{P3})/3\} \times 10^3$]. When CFU/ml of frozen semen was <5000 then that semen was considered satisfactory and used for Artificial Insemination. When CFU/ml of frozen semen was $= 5000$ then semen sample was considered unsatisfactory and that semen should be discarded.

Bacterial identification

Colonies with different morphologies were picked up from each plate for bacterial identification. The pure bacterial isolates were obtained after repeated subculture and identification of the isolates were done by morphological, cultural and biochemical characterization as per standard protocol (Patel *et al.* 2011). The biochemical tests that were performed for tentative identification were Methyl Red test, Voges- Proskauer test, lead acetate paper strip test, urea hydrolysis, nitrate reduction, indole formation, citrate utilization, oxidase and catalase production. However, these conventional biochemical tests were not performed in case of *Corynebacterium* spp. and *Streptococcus* spp. Catalase test was performed for initial differentiation of *Streptococcus* from *Staphylococcus*. Subsequently, haemolytic pattern on sheep blood agar was considered for differentiation between alpha-haemolytic and beta-haemolytic strain of *Streptococcus*. Some fungal colonies were also isolated from the semen samples by culturing on Saboraud

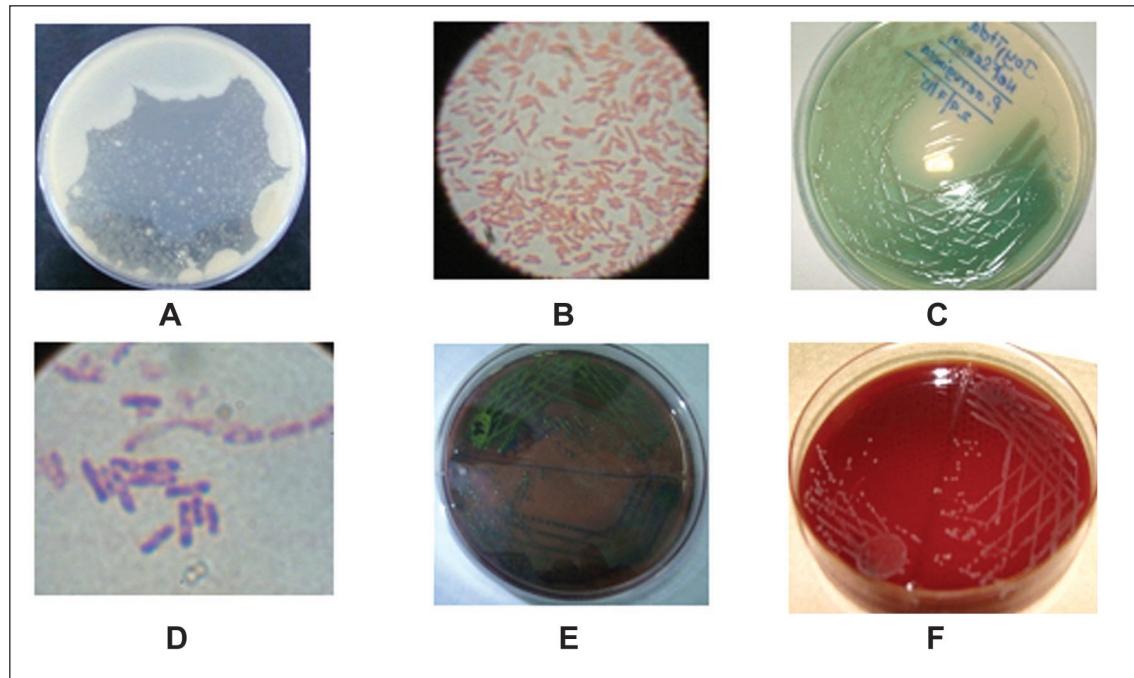


Fig. 1. Morphology and colonial character of different bacterial isolates from frozen semen.

[Culture plates showing growth of fungus (A), morphology of *Pseudomonas aeruginosa* under microscope (B), colony of *Pseudomonas aeruginosa* (C), morphology of *Bacillus* spp. (Anthracoid) (D), colony of *E. coli* on EMB agar (E), colony of *Staphylococcus* spp on Blood Agar (F)].

Dextrose Agar and morphological characterization after staining with lactophenol cotton blue stain was done.

RESULTS AND DISCUSSION

The average bacterial load in frozen semen straw (FSS) from 215 cattle bulls are presented in Table 1. A total of 23 pooled samples (Haringhata - 11, Salboni - 9 and Beldanga-3; $23 \times 4 = 92$ straws) (10.69%) showed bacterial load of more than 5000 CFU/ml and were considered unsatisfactory for artificial insemination (Table 2). The study revealed that 42 (20%) of the frozen semen samples were contaminated either with different bacteria or fungi, which are in agreement with the result

of Najee *et al.* (2012) in imported bulls. Abro *et al.* (2009) isolated and characterized 7 different pathogenic bacteria from 100 frozen semen samples of cattle. The higher level of bacterial contamination may be due to non-aseptic condition during semen collection, processing or resistance to antibiotic used in the extender. All the 23 (10.69%) positive samples were contaminated with both Gram negative and Gram positive bacteria (Fig.1).

Bacterial contaminants in semen have survived at -196°C in liquid nitrogen. Wide variations in number of colony forming unit (CFU) per ml of frozen semen were obtained in the present study (Table 2), which was in agreement with Ahmed *et al.*, (2001) and Shukla

Table 1. Microbiological quality of frozen semen straws.

| Sl.No. | No. of sample | CFU/ml | REMARKS |
|--------|---------------|--------|----------------|
| 1 | 23 | >5000 | Unsatisfactory |
| 2 | 192 | <5000 | Satisfactory |

Table 2. CFU count for microbiological quality assessment in frozen semen straw.

| SL. No. | No. of Sample | CFU/ml | Remarks |
|-------------------------|---------------|---------------------|----------------|
| 1 | 1 | 21X10 ³ | Unsatisfactory |
| 2 | 3 | 17 X10 ³ | Unsatisfactory |
| 3 | 5 | 14 X10 ³ | Unsatisfactory |
| 4 | 6 | 12 X10 ³ | Unsatisfactory |
| 5 | 8 | 10 X10 ³ | Unsatisfactory |
| 6 | 11 | 4 X10 ³ | Satisfactory |
| 7 | 19 | 3 X10 ³ | Satisfactory |
| 8 | 26 | 2 X10 ³ | Satisfactory |
| 9 | 32 | 1 X10 ³ | Satisfactory |
| 10 | 104 | No Colony | Satisfactory |
| Total number of samples | 215 | | |

Table 3. Bio-chemical properties of isolated bacterial species.

| Sl. No. | VP | CU | LA | UH | NR | I | MR | CAT | OXI-DASE | Confirmation of bacterial isolates |
|---------|----|----|----|----|----|---|----|-----|----------|------------------------------------|
| 1 | + | + | - | ± | + | - | + | + | - | <i>Staphylococcus</i> spp. |
| 2 | - | + | - | - | + | - | - | + | + | <i>Pseudomonas aeruginosa</i> |
| 3 | | | | | | | | + | + | <i>Micrococcus</i> spp. |
| 4 | - | - | - | - | + | + | | + | - | <i>E.coli</i> |
| 5 | - | - | + | + | + | ± | | + | - | <i>Proteus</i> spp. |
| 6 | | | | + | + | | | + | - | <i>Corynebacterium</i> spp. |
| 7 | + | + | - | + | + | - | - | + | - | <i>Klebsiella</i> spp. |
| 8 | | | | | | | | | | <i>Anthacoids</i> |
| 9 | | | | | | | | | | <i>Streptococcus</i> spp. |

(2005). In five semen samples fungal colony, characteristic *Aspergillus niger* was detected. On basis of colonial morphology and

biochemical properties isolates were tentatively identified as *Staphylococcus* (11 samples), *Micrococcus* (2 samples), *Corynebacterium*

Table 4. Cultural characteristics and tentative identification of bacteria isolated from frozen semen samples.

| SL. No. | No. of samples | Gram Staining reaction | Growth on special media | Haemolysis pattern | Tentative bacterial identification |
|---------|----------------|---|--|--------------------|------------------------------------|
| 1 | 11 | Gram Positive cocci | Small red colony on MSA | Non-haemolytic | <i>Staphylococcus</i> spp. |
| 2 | 4 | Gram Negative bacilli | Greenish blue pigment on nutrient agar and growth on 2% Dettol Nutrient Agar | Non-haemolytic | <i>Pseudomonas aeruginosa</i> |
| 3 | 2 | Gram Positive cocci ,arranged in tetrad | Blood agar | Non-haemolytic | <i>Micrococcus</i> spp. |
| 4 | 3 | Gram Negative slender rods | Metallic sheen on EMBA | Non-haemolytic | <i>E. coli</i> |
| 5 | 5 | Gram negative slender rods | Black centred colony found on XLD agar | Non-haemolytic | <i>Proteus</i> spp. |
| 6 | 1 | Gram positive small rods with Chinese letter like arrangement | Blood agar | Non-haemolytic | <i>Corynebacterium</i> spp. |
| 7 | 2 | Gram negative slender rods | Lactose fermenting colony on Mac Conkey agar | Non-haemolytic | <i>Klebsiella</i> spp. |
| 8 | 13 | Gram Positive large rods | No growth on Mac Conkey agar | Beta-haemolytic | Anthracoid |
| 9 | 2 | Gram Positive rods with chained appearance | Blood agar | Beta-haemolytic | <i>Streptococcus</i> spp. |

(1 sample), *Pseudomonas* (4 samples), *Proteus* (5 samples), *Escherichia* (3 samples), *Klebsiella* (2 samples), *Streptococcus* spp. (2 samples), *Bacillus* spp. other than *Bacillus anthracis* (13 samples) (Table 3 and Table 4). These organisms have been reported to be predominant among semen microbes. Among the identified bacteria, *Bacillus cereus*

(Andersson *et al.* 2004), *B. licheniformis* (Cabell 2007), *Micrococcus* (Abro *et al.* 2015), *Pseudomonas* (Smole *et al.* 2010), *Streptococci* and *Corynebacterium* have been associated with bovine abortion while *Klebsiella pneumoniae* infection leads to repeat breeding problem and *Staphylococcus aureus* affects metabolic activities of spermatozoa

(Meyer *et al.* 1980). The recovery of *Proteus* sp. was in agreement with the report of Rahman *et al.* (1983) and recovery of *Bacillus* spp. other than *Bacillus anthracis* was corroborate with the findings of Gangadhar *et al.* (1986). The presence of these bacteria is significant as they may compete with spermatozoa for nutrition; alter the physiological behavior of the female reproductive tract leading to failure of fertilization, early embryonic death, abortion and/or repeat breeding. Bacteria produce reactive oxygen species (ROS) in contaminated semen which impair sperm function and reduces its fertilization potential (Morrell 2006). Due to very small sperm cell cytoplasm, the cytosolic anti-oxidants are not enough to counteract with this ROS (Drevius 1970). Sperm motility is significantly affected by bacteria by its adherence to spermatozoa (Diemer *et al.* 1996). Sometimes, microbes can interact with acrosome directly or react through production of toxin (Morrell 2006). It is documented that bacteria in the semen are controlled by using antibiotics in freezing diluents. Conventionally, benzyl penicillin (1000 IU /ml) and streptomycin sulphate (1000mg/ml) alone or in combination, chloramphenicol and sulphonamide, are usually added to the semen extender (Akhter *et al.* 2008). Toxic or deleterious effects of antibiotics on spermatozoa may be evaluated for their use in cryopreservation of bovine semen.

CONCLUSION

The present study estimated presence of different bacteria in frozen semen samples in spite of adding different combination of antibiotics in semen extender during processing. Further works are required to examine antibiotic resistance of the isolates and to find safe and

better effective antibiotic to be added to semen during preservation. Further, stringent hygienic measures are also required before as well as during semen collection and processing.

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